

<b>REPORT DOCUMENTATION PAGE</b>				Form Approved OMB No. 0704-0188	
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1. REPORT DATE (DD-MM-YYYY) 01-03-2009		2. REPORT TYPE Final Report		3. DATES COVERED (From - To) 01-04-2008 to 01-03-2009	
4. TITLE AND SUBTITLE  AFSOR Bio-X: Encapsulated DNA-Based Molecular Autonomous Sensing Devices With Photonic I/O				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER FA9550-08-1-0188	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S)  John H Reif and Erik A. Schultes				5d. PROJECT NUMBER	
				5e. TASK NUMBER 01	
				5f. WORK UNIT NUMBER 01	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Dept of Computer Science, Duke University, Durham, NC 27708				8. PERFORMING ORGANIZATION REPORT NUMBER 01	
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES) AFRL/AFOSR, 875 N. Randolph St. Suite 325, Rm 3112, Arlington, VA 22203-1768				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S) 01	
12. DISTRIBUTION/AVAILABILITY STATEMENT  A					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT					
15. SUBJECT TERMS  DNA, detection					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			19b. TELEPHONE NUMBER (Include area code)



**Final Report (March 1, 2009) AFSOR Grant: FA9550-08-1-0188**  
**AFSOR Bio-X: Encapsulated DNA-Based Molecular Autonomous Sensing Devices With Photonic I/O**

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**Goal:** DNA-based nanostructure detector system development and demonstration with multispectral optical readouts. The overarching goal of this research was the remote hyperspectral detection of molecular targets, with application as a combat ready, modular, general purpose target detection and signal amplification system coupled to airborne imaging systems. The specific Tasks of this project were:

- (1) To develop systems that amplify detection events through the use of existing biomolecular amplification methods;
- (2) To optimize hydrogel/aerogel encapsulation and preservation of the biomolecular detection systems to ensure robust activity upon rehydration in the field;
- (3) To demonstrate remote detection by such systems using hyperspectral imaging.

**Project Start Date: April 2008**

**Summary of Final Status in Spring, 2009:**

Erik A. Schultes & John H. Reif at Duke have worked on the three specific Tasks. Multispectral optical readouts were obtained for two DNA-based nanostructure detector systems for three DNA analytes, including unique genomic identifiers for two human pathogens. Initial studies in gel encapsulation were conducted as well.

**Overview:**

Previously, our team implemented experimental demonstrations at Duke of two distinct modes of DNA-based nanostructure detectors for arbitrary DNA analytes. In Detection Method 1, the detector system was initially conceived to consist of two, and in some cases three, distinct DNA strands that, upon contact with the analyte, undergo an autocatalytic hybridization chain reaction resulting in a very high molecular weight dendritic nanostructure. The dendritic nanostructures are configured to organize appropriately labeled gold nanoparticles, inducing a plasmonic resonance and a concomitant shift in optical absorbance near 520 nm.

In Detection Method 2, a similar DNA hybridization system was used to trigger an autocatalytic opening of circularized deoxyribozymes. These linear products are configured to organize appropriately labeled gold nanoparticles as before. Both systems are capable of exponential reaction kinetics (working in minutes) to produce naked eye readouts of sub-micromolar amounts of DNA analyte. The key technical goals of our work this FY2008 have been to optimize these molecular detection systems to provide the highest possible fidelity, with specific goals of reproducibility and the elimination of false-positive outputs.

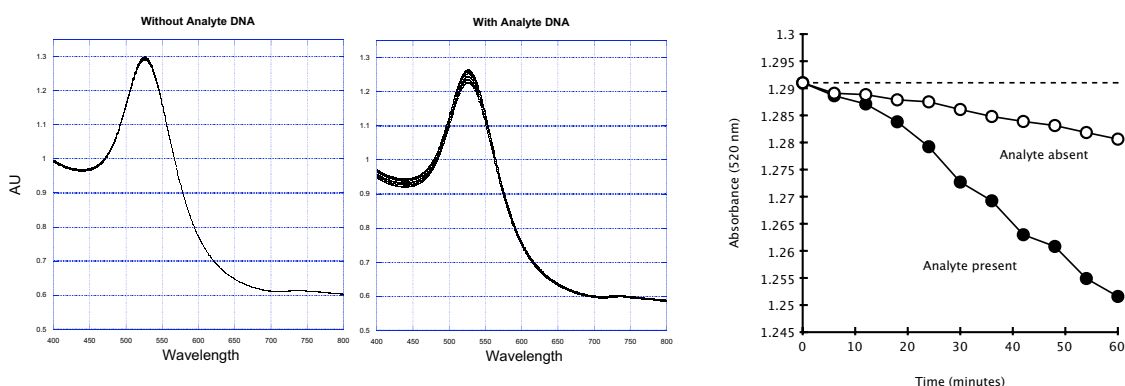
In 2008 we focused our experimental efforts on the DNA annealing conditions (quenching in water), DNA storage conditions ( $-20^{\circ}\text{C}$ , not  $4^{\circ}\text{C}$ ), maintaining low divalent cation concentrations (even in analytical gels), running numerous negative controls and defining finite reaction-time windows. We report advancements regarding three elements relating to both Detection Methods.

**Details of Work Done:****(1) Routine Optical Readouts**

First, we have optimized and established a highly reproducible and sensitive gold nanoparticle optical readout protocol. Following these improved methods, multispectral shifts in absorbance properties (from 450 - 800 nm) of gold nanoparticles, indicative of analyte DNA detection, are now routine.

Following an in-person visit to Dr. John Nolan's laboratory at the La Jolla Bioengineering Institute (January 27 - February 5), and working closely with Dr. David Sebba (a graduate of Duke and expert with gold nanoparticle optical readout methods), we were able to establish a reliable protocol for both of our Detection Methods. Parameters that were key to increasing the reproducibility of the optical readout included the chemistry of the DNA-gold nanoparticle functionalization, considerations of the topological configurations of DNA-gold nanoparticle molecular interactions (i.e., so-called "head-head", "head-tail", "tail-tail" configurations), and finding proper concentrations of gold nanoparticles for spectrophotometric analysis.

Having optimized these parameters, we proceeded to demonstrate, through a series of carefully controlled test reactions, a reliable and reproducible spectral shift indicative of controlled gold nanoparticle aggregation. We then demonstrated comparable results with the three-strand HCR Detection Method. Specifically, we combined 1 micromolar amounts of the three-strand system (alpha + beta + gamma) with appropriately labeled gold nanoparticles, with and without the analyte DNA. We then monitored the optical absorbance of the system (400 - 800 nm) with time. Using this method, we successfully detected 1 picomole of analyte DNA (10 micoliter volume) based on shifts in absorbance spectra that began within minutes but continued for more than one hour. Plotted below are the raw spectra and the normalized spectral shifts with reliable detection (i.e., more than 2-fold differences between the control and target reactions) occurring within 25 minutes.

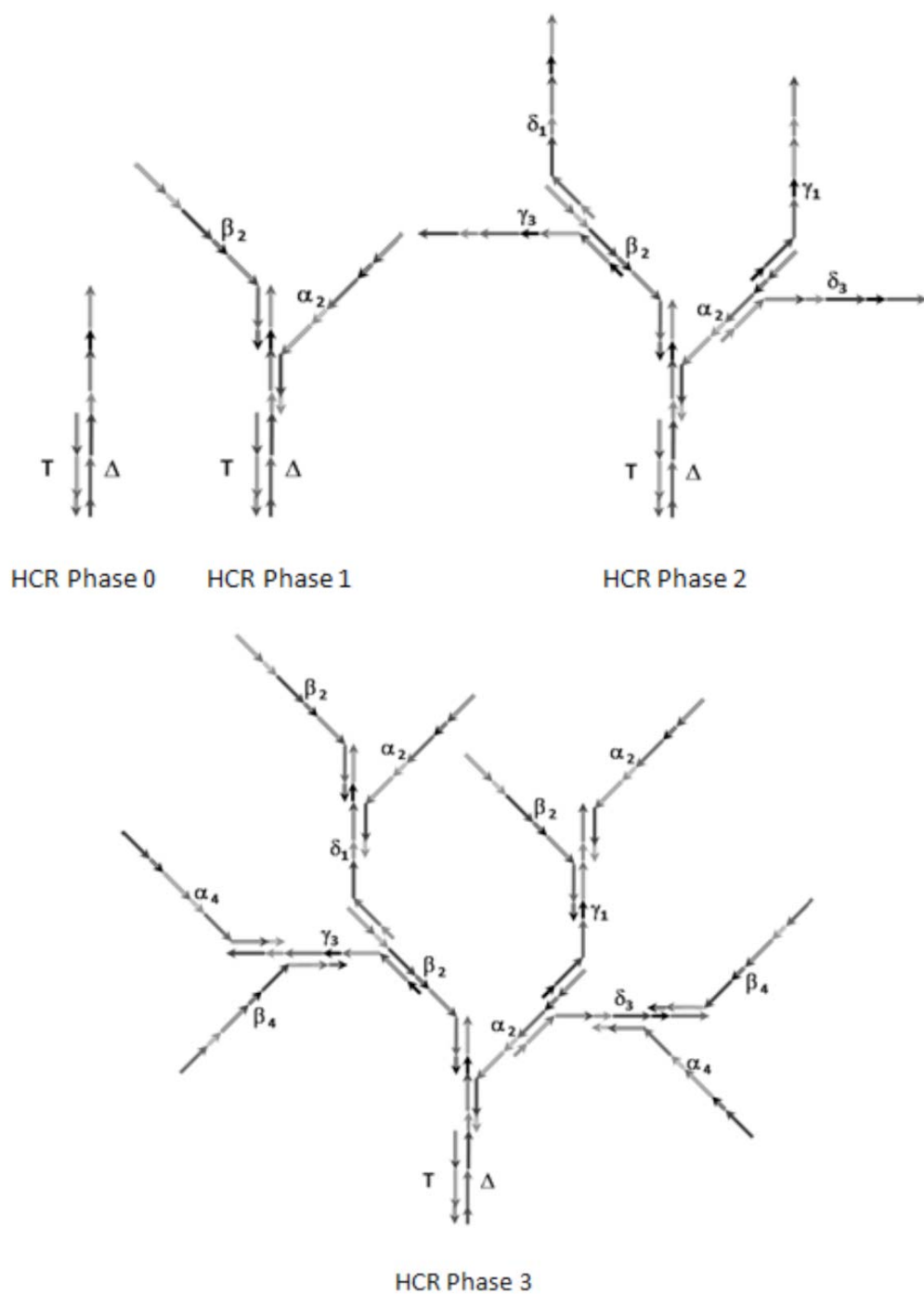


#### (4) Sequence and Structural Optimization of Dendritic Nanostructures

The HCR Detection Method results in the recursive, sequence-specific growth of a complex dendritic nanostructure. Although in principle, the number of branches in the structure should grow exponentially in time, in practice the system results in a spectrum a products that are finite in size. This practical limitation is most likely due to steric hinderances that progressively confound the binding of DNA stands in each successive phase of dendromere growth. We believe that these steric limitations are the primary factor in false positive readouts and that if they could be resolved, would increase the sensitivity of the Detection Method.

Working in close collaboration with the Information Directorate, Air Force Research Laboratory, Rome, New York (Morgan A. Bishop and Clare D. Thiem), we undertook an intriguing analysis whereby three classes of putative steric hinderances were identified and mitigated through carefully designed sequence modifications. The resulting modifications involve additional DNA strands which have the unwanted effect of making the Detection Method more complex in its preparation, but the system was explored here in the interest of

learning more about this novel class of DNA nanostructure and in optimizing the sensitivity of the readout. A schematic of the predicted dendritic growth is shown below:



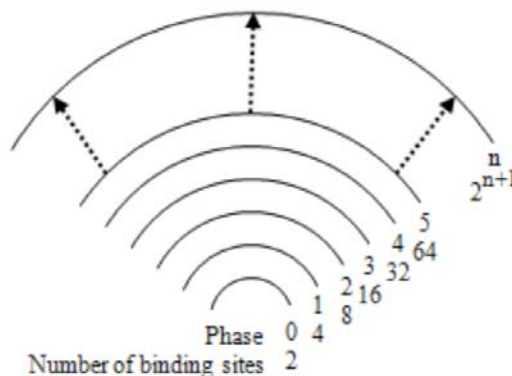
### Modification 1:

Our first modification was intended to reduce the false positive rate by creating a specific external toehold for the initiator hairpin. The analyte to be detected, denoted T, systematically interacts with only this initiator strand, denoted  $\Delta$ , during the target recognition phase of the reaction. Thus, the toehold length may be optimized to maximize signal-to-noise ratio without negatively affecting the HCR amplification phases. In addition, the concentration of this hairpin establishes a tree-to-branching ratio which is related to the expected number of growing trees within a reaction versus the expected amount of branching within a tree. We can predict the average amplification within a tree using the ratio  $\Delta:H$ , where  $\Delta$  and H are the concentration of the initiator strand and all other hairpin strands, respectively:

$$\text{Average Amplification} = \frac{2H}{\Delta}$$

### Modification 2:

The second modification helps achieve exponential amplification beginning at the first stage of the reaction and creates more equally stable hairpin structures before a HCR begins. Exponential amplification is achieved by creating two amplification binding sites, one which is interior (IBS) and one which is exterior (EBS), immediately following the target recognition trigger (Phase 0 in schematic). Each of these sites were then be amplified exponentially throughout the HCR. See figure below:



More equally stable hairpins, or those with similar melting temperatures, help ensure that the different types of hairpins have similar reaction rates regardless of environmental conditions. With these proper hairpins, poor tree growth occurs at the consequence of the entire system as opposed to an individual component. Clearly, systematic failure is a feature which is easier to detect experimentally than single component failure. Thus, error rates associated with environmental conditions are more stable and error recognition can be deciphered more readily.

### Modification 3:

The third modification alleviates an identified potential structural issue while minimizing the required number of extra distinct hairpins. The prevention of this structure requires four additional hairpins, but they are products of the same DNA sub-strands that exist in the previous modification's hairpins. If this structure being prevented was to arise merely once in a single dendritic tree it would cut any future potential signal amplification from that branch site in half. The structure that this modification prevents is shown below:



With all three modifications employed, we predict a more structurally sound dendritic tree recursive reaction. Table 1 shows the new phase growth with respect to the specific hairpin binding sites. These sites may then be targeted by signal emitters such as flourophores or gold nanoparticles.

Table 1. Phase growth with respect to number of specific hairpin binding sites

		Phase								
		0	1	2	3	4	5	6	7	8
Sites	bc*	1		2		8		32		128
	ad*	1		2		8		32		128
	ca*		1		4		16		64	
	db		1		4		16		64	
	db*		1		4		16		64	
	ca		1		4		16		64	
	b*c*			2		8		32		128
	a*d*			2		8		32		128

### Experimental Implementation of the Three Modifications:

Using our existing three-strand dendritic nanostructure system for detecting the human pathogen *Chlamydia trachomatis*, *Ct*, as a standard, we implemented and compared the kinetic behavior of the three modifications described above for the same analyte DNA. The three modifications were examined as two experiments: modifications 1 and 2 were combined as a single experiment (a six-strand system), then modifications 1, 2, and 3 were combined as a single experiment (a nine-strand system). The separate stands were annealed by heating to 90C for 1 minute and cooling to room temperature on the bench over 10 minutes (tubes were centrifuged to eliminate condensation). In all cases, each strand species was present at 1 micromolar while analyte was present at 1/10 this amount. The reaction was





By quantifying the band intensities (AlphaImager 4.1.0) the two system modifications demonstrate clear differences in behavior relative to the original control system. The overall fraction reacted increased for the second modification (2 to 3-fold increase) but was nearly the same for the third modification. As the over-all amount of DNA in the third modification is higher than in the control reaction (given a comparable reaction assembly protocol we implemented herein), this means that the absolute quantity of product DNA is higher for the third modification.

However, for both the second and third modifications, the majority of the reacted DNA was localized to the loading wells (indicating products of very-high molecular that are larger than the pore size of the gel matrix and unable to migrate into the gel) whereas the control reactants resolve into a spectrum of distinct lower molecular weight products (i.e., the ladder under the control *Ct* + lane, not seen in the modification lanes). From this head-to-head comparison, we may conclude that despite the increased number of strands in the modified systems, the intended sequence optimizations of the original control system function to produce more product having a higher-molecular weight. These results suggest that such modifications could lead to increased detection sensitivities.

### **Collaborations with AFRL BIOX Teams:**

We discussed with the HPW/RHPC (PI: Kramer) BioX team on the planned experimental work on Task 2: initiation of experimental tests of hydrogel/aerogel encapsulation, and detection optimization and optical readout after rehydration.

Two meetings with the Rome Team at Duke in FY 2008 facilitated initial work by the AFRL/RI (PI: Renz) BioX team on development of software for design and optimization of DNA sequences used in these protocols for specified molecular targets to be detected, and development of kinetic simulation software, as well as provided valuable ideas for further optimization of detection protocols.

**Publications:**

These results are planned to be published separately, as two manuscripts in *Nature Nano* and *JACS*. The manuscripts drafts have been prepared as submitted:

(1) Thomas H. LaBean, Geetha Shetty, Peng Yin, Erik A. Schultes, John H. Reif, A Dendritic Nanostructure for DNA Detection, to be submitted to *Nature Nano*.

(2) Thomas H. LaBean, Geetha Shetty, Hao Yan, Erik A. Schultes, John H. Reif , Target DNA Detection by Strand Displacement and Deoxyribozymogen Amplification, to be submitted to *JACS*.